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Effects of Extracts of *Zanthoxylum* Fruit and their Constituents on Spontaneous Beating Rate of Myocardial Cell Sheets in Culture

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In the course of our studies on naturally occurring cardioactive agents, we investigated the effects of water and methanol extracts of a Chinese crude drug 'Huajiao' the dried fruit of *Zanthoxylum bungeanum*, on the spontaneous beating rate (BR) of embryonic mouse myocardial cell sheets in culture. Both extracts significantly increased the BR. Through bioassay directed fractionation of the extracts, hydroxy-β-sanshool (1b), xanthoxylin (2) and two quercetin glycosides, hyperin (4) and quercitrin (6), were found to increase the BR in a standard medium (2.1 mM Ca^{2+}). In a low Ca^{2+} medium (0.5 mM Ca^{2+}), these compounds suppressed the decrease of BR, which was induced by low Ca^{2+}. Of 16 flavonoids related in structure with hyperin (4) and quercitrin (6), quercetin, isoquercitrin, rutin, myricetin and myricitrin also increased the BR in the standard medium, while kaempferol and luteorin decreased the BR in the standard medium. When compared with control, hydroxy-β-sanshool (1b) and xanthoxylin (2) stimulated 13–15 fold calcium uptake of the cultured myocardial cells, which might have caused the positive chronotropic effect. Hyperin (4) and quercitrin (6) did not affect calcium uptake of the myocardial cells, Na^+–K^+ ATPase activity or Ca^{2+}-ATPase activity of sarcoplasmic reticulum.

Keywords: cultured myocardial cell; positive chronotropic effect; spontaneous beating; *Zanthoxylum bungeanum*.

INTRODUCTION

'Huajiao' (Kasho in Japanese) is a traditional Chinese crude drug derived from the fruit of *Zanthoxylum* plants. The crude drug has been used as a stomachic, an antiphlogistic, a diuretic, a vermifuge, as well as a spice and a seasoning. Local stimulative effects of a water extract of 'Huajiao' on digestive organs and its local anaesthetic action on animal skins have been reported in recent years (Mizutani *et al.*, 1988).

In the course of our studies to find new cardioactive substances from natural sources (Huang *et al.*, 1987, 1990, 1991a,b; Namba *et al.*, 1988, 1989; Kakiuchi *et al.*, 1991) we have screened various Chinese crude drugs using an *in vitro* myocardial cell system and found that a water extract of 'Huajiao' stimulated the spontaneous beating rate (BR) of myocardial cells in culture (Huang *et al.*, 1987).

In the present paper, we describe further studies on the stimulative effect of water and methanol extracts of 'Huajiao' on the BR, and on isolation of cardioactive principles from the extracts through bioassay directed fractionation.

MATERIALS AND METHODS

Apparatus. Melting points were measured with a Yanagimoto melting point apparatus (Yanagimoto Co., Kyoto, Japan) and are not corrected. Nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ containing tetramethylsilane (TMS) as internal standard with JEOL GX-270 (^{1}H-NMR, 270 MHz) and FX-90Q (^{13}C-NMR, 22.5 MHz) NMR spectrometers (JEOL, Co., Akishima, Japan). Mass spectra (MS) were measured in an electron impact (EI) mode at an ionization voltage of 70 eV with a JEOL DX-300 mass spectrometer. Fast atom bombardment (FAB) mass spectra were carried out with glycerol as the matrix. High-performance liquid chromatography (HPLC) was carried out with a JASCO Tri Rotar V liquid chromatograph equipped with a JASCO UVIDEC-100 UV spectrophotometer and a Shimadzu C-R6A chromatopac. Calcium, potassium and sodium contents were determined with an atomic absorption spectrometer (Shimadzu, Kyoto, Japan).

Plant materials. Two types of commercial products of 'Huajiao' were purchased from Tochimoto Tenkaido Co. (Osaka, Japan). They were produced in Sichuan and Shangdong of China, and are deposited in the Herbarium of Materia Medica of Toyama Medical and Pharmaceutical University. The botanical source of both products was identified as the dried fruit of *Zanthoxylum bungeanum* Maxim.

Chemicals and media. Calcium-containing and calcium-free Eagle's minimal essential medium (Eagle's MEM) and Dulbecoo's calcium- and magnesium-free phosphate buffered saline (PBS(−)) were products of Nissui Laboratories (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Whittaker M&A Bioproducts (Walkersville, USA); *N*-2-hydroxyethylpiperadine-*N'*-

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2-ethanesulphonic acid (HEPES) from Dojin Laboratories (Kumamoto, Japan); ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*, *N*′,*N*′-tetraacetic acid (EGTA) and ouabain from Wako Pure Chemical Industries Ltd (Osaka, Japan). $^{45}CaCl_2$ (0.074 MBq/mL) from Du Pont Co. (Wilmington, USA); adenosine 5'-triphosphate (ATP), Na^+-K^+ATPase and A 23187 from Sigma Co. (St. Louis, USA); standard solutions of Na^+, K^+ and Ca^{2+} from Nacalai Tesque Inc. (Osaka, Japan) and Wako Pure Chem. Ind. Ltd; quercitrin, isoquercitrin, pelatoside, rhamnetin, morin, tamarixetin, ombulin, fisetin, luteolin 7-glucoside and kaempferide from Extrasynthese (Genay, France); myricitrin, luteolin and eriodictyol from Carl Roth (Karlsruhe, FRG); rutin and kaempferol from Wako Pure Chem. Ind. Ltd. Trypsin and collagenase were products of Difco Co. (Detroit, USA) and Sigma Co. (St. Louis, USA), respectively. Bidistilled water was obtained from Flow Laboratories Inc. (New South Wales, Australia). Other reagents used were of a special grade.

Preparation of myocardial cell sheets. Cultured myocardial cells were prepared by the methods of Goshima and Tonomura (1969) and Namba *et al.* (1988). The heart taken from 14–16 day old embryo of ICR mouse (Sankyo Laboratories, Tokyo, Japan) was digested with a mixture of collagenase (0.025%; 77 units/mL) and trypsin (0.125%; 312.5 units/mL) in PBS (–) for 10 min at 37 °C. The same volume of Eagle's MEM supplemented with 10% FCS (standard medium) was added to stop the digestion. The cells were collected by centrifugation at 200 × g and resuspended in the standard medium. Fibroblast-like cells were removed according to the method of Polinger (1970). The cells were then seeded onto a glass cover strip coated with fibronectin in a 35 mm plastic Petri dish, and incubated at 37 °C under an atmosphere of 5% CO_2–95% air for 48 h. The cells were clustered on the fibronectin-coated portion and a single cell layer was formed.

Measurement of the beating rate (BR) of myocardial cell sheets. A cell sheet attached to the cover glass was mounted upside down on a small chamber in an acrylic plate (2 mm thick). The chamber was filled with a standard medium for 5 min. The motion associated with the contraction and relaxation of the cell sheet was observed under a phase-contrasted microscope (Olympus IMT-2) at 37±1 °C. The beating rate was monitored by a photoelectric recording method (Namba *et al.*, 1988). Under the conditions, the BR was constant with average 140±15 (SE) beats/min ($n = 9$) and this value was adopted as an initial BR (100% at time 0). The medium was then replaced with a standard or low calcium medium, containing a test sample dissolved in dimethyl sulphoxide (DMSO; the final DMSO concentration was 1% of the medium). The beating rate was measured for 15–20 min after replacement of the medium. Control experiments were carried out in the medium containing 1% DMSO without the test sample.

Preparation of a MeOH extract of 'Huajiao' and its fractionation. Three kg of a pulverized crude drug, 'Huajiao' produced in Shandong were extracted ten times with boiling MeOH (1.3 L each) for 3 h for each extraction. The combined MeOH solutions were evaporated *in vacuo* to give a residue (578 g). The MeOH extract was suspended in water (700 mL) and extracted five times with ether (2.0 L each). The combined ether solutions were concentrated *in vacuo*, and the concentrate (222 g) was redissolved in MeOH (500 mL) and extracted with hexane (2.0 L × 5). The hexane-soluble portion was concentrated and filtered to give a precipitate (32 g). On crystallization of the precipitate from MeOH, xanthoxylin (2) was obtained in a yield of 20 g, while the filtrate (119 g by dry weight) was subjected to column chromatography on silica gel with hexane containing an increasing amount of $CHCl_3$ to give mikanin (**3**; 1.2 g). The MeOH-soluble portion (46.9 g) was applied to a silica gel column (5.5 × 84 cm), which was then eluted with hexane-$CHCl_3$ to give a mixture of hydroxysanshools (ca. 100 mg). The mixture was separated into hydroxy-α-sanshool (**1a**; 5 mg) and hydroxy-β-sanshool (**1b**; 50 mg) by HPLC under the following conditions: column, ODS-5 (4 × 150 mm; Nomura Chem. Co., Seto, Japan); flow rate, 1.3 mL/min; mobile phase, CH_3CN–H_2O (1:1); detection, 258 nm. The aqueous solution remaining after the ether extraction was extracted with BuOH (1.5 L × 5) to give BuOH-soluble (344 g) and water-soluble (10 g) fractions.

Preparation of a water extract of 'Huajiao', and its fractionation. A pulverized crude drug of 'Huajiao' (3.0 kg) produced in Sichuan was extracted six times with boiling water (3.0 L each) for 3 h for each extraction. The solutions were filtered and the filtrate was concentrated *in vacuo* to a volume of 1.5 L, which was extracted with ether (7.5 L) to give ether-soluble (14 g) and water-soluble (260 g) fractions. The ether-soluble fraction was applied to a polyamide column (4 × 75 cm) and eluted with MeOH to give fractions. From these fractions, hyperin (**4**; 25 mg), isovanillic acid (**5**; 300 mg) and quercitrin (**6**; 12 mg) were obtained by column chromatography on Diaion HP-20 (Nippon Rensui Co., Tokyo, Japan; column size, 4 × 70 cm; eluting solvent, MeOH) and crystallization.

Hydroxy-β-sanshool (1b). EI-MS *m/z*: 263 $[M]^+$; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 6.86(1H, dt, $J = 15.4$, 6.6 Hz, H-3), 6.05–6.16(5H, m, H-7, 8, 9, 10, NH), 5.83(1H, d, $J = 15.4$ Hz, H-2), 5.69(1H, dq, $J = 13.9$, 6.8 Hz, H-11), 5.63(1H, dt, $J = 14.2$, 7.3 Hz, H-6), 3.32(2H, d, $J = 6.1$ Hz, H-1′), 2.25(4H, m, H_2-4,5), 1.77(3H, d, $J = 6.6$ Hz, H_3-12), 1.25(6H, s, H_3-3′,4′); the ^{13}C-NMR spectral data agreed with those reported (Mizutani *et al.*, 1988).

Xanthoxylin (2). EI-MS *m/z*: 196 $[M]^+$; ^{1}H-NMR (270 MHz, $CDCl_3$): δ 6.03(1H, d, $J = 2.4$ Hz, H-3), 5.90(1H, d, $J = 2.4$ Hz, H-5), 3.84, 3.80 (each 3H, s, MeO x2), 2.59(3H, s, Me); the ^{13}C-NMR spectral data agreed with those reported (Wagner *et al.*, 1976).

Mikanin (3,5-dihydroxy-4′,6,7-trimethoxyflavone) (3). EI-MS *m/z*: 344 $[M]^+$, 329$[M-15]^+$; ^{1}H-NMR(270 MHz, DMSO-d_6): δ 8.17(d, 2H, $J = 8.8$ Hz, H-2′,6′), 7.15(2H, d, $J = 8.8$ Hz, H-3′,5′), 6.58(1H, s, H-8), 3.92(3H, s, MeO), 3.85(3H, s, MeO), 3.82(3H, s, MeO), 12.26(1H, s, HO-5), 9.64(1H, s, HO-3); the ^{13}C-NMR spectrum agreed with that reported by Jauhari *et al.* (1979).

Hyperin (4). FAB-MS *m/z*: 465[M+1]$^+$, 303[M+1-galactose]$^+$; ^{1}H-NMR (270 MHz, DMSO-d_6): δ 7.66(1H, dd, J=8.5, 2.2 Hz, H-6'), 7.53(1H, d, J=2.2 Hz, H-2'), 6.82(1H, d, J=8.5 Hz, H-5'), 6.40(1H, d, J=2.1 Hz, H-8), 6.20(1H, d, J=2.1 Hz, H-6), 5.38(1H, d, J=7.6 Hz, H-1''), 3.65-3.17 (sugar protons); the ^{13}C-NMR spectrum agreed with that reported by Markham and Chair (1982).

Isovanillic acid (5). EI-MS *m/z*: 168[M]$^+$; ^{1}H-NMR (270 MHz, DMSO-d_6): δ 7.41(1H, dd, J=8.3, 2.2 Hz, H-6), 7.35(1H, d, J=2.2 Hz, H-2), 6.98(1H, d, J=8.3 Hz, H-5), 3.82(3H, s, MeO-4).

Quercitrin (6). FAB-MS *m/z*: 449[M+1]$^+$, 303[M+1-rhamnose]$^+$; ^{1}H-NMR (270 MHz, DMSO-d_6): δ 7.30(1H, d, J=2.2 Hz, H-2'), 7.25(1H, dd, J=8.3, 2.2 Hz, H-6'), 6.86(1H, d, J=8.3 Hz, H-5'), 6.39(1H, d, J=2.0 Hz, H-8), 6.20(1H, d, J=2.0 Hz, H-6), 5.26(1H, d, J=1.7 Hz, H-1''), 4.94-3.10 (sugar proton), 0.82(3H, d, J=5.5 Hz, H-6''); the ^{13}C-NMR spectral data were in agreement with those reported by Markham and Chair (1982).

Preparation of sarcoplasmic reticulum (SR). Crude sarcoplasmic reticulum fraction from a rabbit heart was prepared by Chamberlain's method with modification (Chamberlain *et al.*, 1983): the heart was taken from a male rabbit (2.5 kg) anaesthesized with pentobarbital and rinsed with ice-cold 0.145 M NaCl to remove blood. All the following operations were carried out at 0–5 °C. The isolated heart was sliced and weighed, and then homogenized in 5 volumes (v/w) of solution A (0.25 M sucrose, 0.5 mM DTT, 3 mM NaN_3, 10 mM imidazole-HCl (pH 6.8)) for 30 min using a teflon homogenizer. The homogenate was centrifuged at 3800×g for 15 min. The resulting supernatant was filtered through 5–7 layer gauze and centrifuged at 119 200×g for 120 min. The pellet was suspended in 2 volumes of solution A supplemented with 0.65 M KCl, allowed to stand for 30 min and then centrifuged at 4400×g for 10 min. The supernatant was again sedimented at 250 000×g for 100 min. The final pellet was suspended in a small volume of solution A and stored at −50 °C.

Measurement of sodium, potassium and calcium contents. Contents of inorganic elements were determined by atomic absorption spectrometry. Na^+, K^+ and Ca^{2+} contents were 16.5, 94.9 and 10.8×10^{-5} mol/g (dry weight), respectively, for the water extract of 'Huajiao', while their contents were 3.6, 1.5 and 0.9×10^{-5} mol/g, respectively, for the MeOH extract.

Measurement of calcium uptake by cultured myocardial cells. Calcium uptake was measured according to the previously reported method (Kakiuchi *et al.*, 1991) and protein analysis was done by the method of Lowry *et al.* (1951).

Effects of compounds on adenosine 5'-triphosphatase (ATPase) activity. ATPase activity was measured with inorganic phosphorus (P_i) release from ATP by the method of Ottolenghi (1975). Calcium-dependent ATPase (Ca^{2+}-ATP) activity was determined as the difference in P_i release between the reaction with 50 mM $CaCl_2$ and that with 1 mM EGTA (Shoshan and MacLennan, 1981). ATPase reaction was carried out for 30 min at 37 °C in a mixture (0.1 mL) containing 1 mg/mL of SR protein, 0.1 M KCl, 3 mM $MgCl_2$, 3 mM ATP (Tris salt), 3 mg/mL of A 23187, 50 mM histidine HCl (pH 7.4) and a test sample dissolved in DMSO. The final DMSO concentration was 1%.

Effects of compounds on Na^+-K^+ ATPase activity. Na^+-K^+ ATPase activity was measured by the methods of Jones *et al.* (1979). A mixture containing 50 mM histidine, 3 mM $MgCl_2$, 1 mM Tris/EGTA, 100 mM NaCl, 10 mM KCl, 3 mM Tris/ATP (pH 7.4) and a test sample dissolved in DMSO (the final DMSO concentration was 1%), was incubated for 60 min at 37 °C, and released inorganic phosphate was determined by the method of Ottolenghi (1975).

Statistical analysis. The data are shown as mean±SEM and statistical significance was evaluated by the Dunnett method.

RESULTS

Effects of extracts of 'Huajiao' on the BR of cultured myocardial cell sheets

By using a cultured myocardial cell system, effects of extracts of 'Huajiao' produced in different areas of China were investigated. Methanol and water extracts of 'Huajiao' from Shangdong, which were dissolved at a concentration of 0.2 mg/mL in a standard medium containing 1% DMSO, significantly increased the BR of embryonic mouse myocardial cell sheets, when compared with control (1% DMSO) (Fig. 1). Under the same experimental conditions, 1% DMSO induced no appreciable inotropic and chronotropic effects on the spontaneous beating. Ouabain at a concentration of 0.1 mM increased the BR by 9–13%, and the methanol

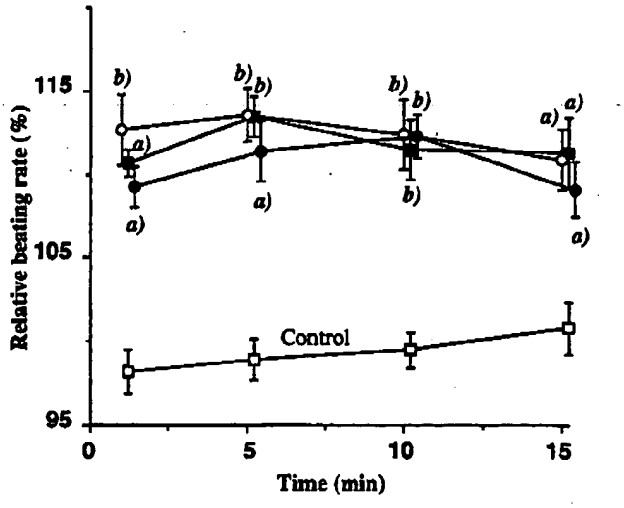

Figure 1. Effects of MeOH and water extracts of 'Huajiao' on the BR of myocardial cell sheets in a standard medium (2.1 mM Ca^{2+}). Concentrations of the extracts and ouabain were 0.2 mg/mL and 0.1 mM, respectively. Control experiments (□) were carried out in a standard medium containing 1% DMSO. Values are expressed as mean±SEM. (n=6). Significantly different from control: ^{a} $p<0.05$; ^{b} $p<0.01$. MeOH extract (■); water extract (○); ouabain (●).